Potential for fungal Bioremediation of Butte Pole Yard Dioxin Contamination

Clifford Bradley, July 2018

This report covers the 4 project tasks outlined below

1. Review 2017 Fourth Five Year Review and Final Dioxin Review and DEQ any other DEQ information or documents supporting conclusions re WRF remediation.
2. Conduct and summarize literature review of WFR remediation of dioxins with focus on field applications.
3. Provide a report based on review of DEQ documents, literature review and my experience including:
    - Estimates/ discussion of complexities, timelines, and uncertainties of WRF remediation
    - Rough estimate of cost for WRF culture needed for treatment,
    - Estimate discuss potential neighborhood Impacts
4. Propose detailed protocol and cost for site specific WRF treatment study

Review DEQ documents

Memo Montana Pole and Treating Plant – More Consideration and Evaluation of Alternatives

The DEQ Memo concluded that "there are three key reasons why WRF is still considered an emerging technology:
1. Extremely low dioxins cleanup levels, often below 1 part per billion or 1 microgram per kilogram (ug/kg) are hard to achieve;
2. The variability of soil conditions found in the field is hard to replicate in the laboratory;
3. The cultivation and delivery of WRF is expensive."

DEQ is confident that it has thoroughly and carefully considered all available treatment alternatives for the Montana Pole Site, including bioremediation. In doing so, DEQ accomplishes the intent of this memo:
1. validate, explain and communicate that dioxin cannot be "re-treated" and remediated any further to meet Montana Pole cleanup levels;

2. validate, explain and communicate that containment (capping) will provide a protective solid barrier between buried, off-loaded/treated soils containing dioxin and the surface and its everyday users.

The Memo included the supplemental document Initial Alternatives Screening Table S W Sawmill Facility FINAL September 19, 2017 containing a Comprehensive List of Potential Treatment Technologies. The list included both In Situ and Ex Situ bioremediation of contaminated soils but does not explicitly include Ex Situ treatment using bioaugmentation with White Rot Fungi (WRF) as a potential alternative. Given the Butte site characteristics, especially depth of soil contamination, treatment would be WRF bioaugmentation of EX Situ

soils: excavated soils would be treated with externally grown cultures of WRF on a substrate such as straw or wood chips most likely in an aerated biopile.

The Memo supported these conclusions with four references regarding dioxin degradation/remediation by white rot fungi:

Richard T. Lamar et al EarthFax Development Corp Evaluation of Fungal-Based Remediation or Treatment of a PCP/Dioxin/Furan-Contaminated Soil from several former Wood-Treating Facilities in New Zealand. In lab scale system using soil from contaminated sites the study screened five species of white rot fungi for dioxin degradation. Direct comparison with the Butte site and conclusion regarding meeting cleanup target of 100ng/kg soil is difficult as the New Zealand soils contained dioxin concentrations more than 100 times greater than the highest concentration in Butte (135,000 to > 1,000,000 ng/kg as HpCDD and OCDD compared to Butte as TEQ 900 to 9,100ng/kg.) One strain (a US isolate of *Pleurotus ostreatus*) reduced HpCDD, OCDD and HpCDF by 82%, 98 and 99% at 56 days treatment. Lower starting concentrations and or longer treatment times might have further reduced concentrations.

Richard T Lamar EarthFax Development Corporation. Large-scale production and field testing of pelleted fungal inocula for use in fungal-based remediation of contaminated soil This study included results of lab tests screening WRF species in PAH contaminated soils from a site in Florida and field scale application of the selected fungus at a PCP/dioxin contaminated site in Montana, the Darby site. The Florida soil contained more than 29,000 mg/kg or nearly 3% PAH. One strain reduced this by 58.5%. For the field test, this strain was cultured on 5 tons of specially designed sawdust based pellets. Because of the change in sites the WRF culture was stored refrigerated for one month then transported cross country and used to inoculate soil in aerated bio piles at the Darby MT site. Treatment reduced PCP in the soil by up to 89% and Dioxin as TEQ by 31%. The fungal strain was selected for PAH degradation in a soil from a site in Florida and never evaluated in lab scale culture for dioxin degradation in the MT site.

This study also included estimated cost for the fungal culture and is the source of the $5 million cost estimate in the Memo for WRF treatment of the Butte site. The study estimated total cost for pelleted culture at $560/ton or $28/ton of treated soil at a 5% w/w application rate of culture in soil. The cost for pelleted culture included $86.75/ton of culture for substrate, and packaging plus $60/ton for transport 1,500 miles to the Montana site. Estimating cost for fungal culture produced for the Butte site would require a detailed analysis, however in our experience large scale fungal culture produced locally in bulk using local substrate such as wood chips and without packaging would cost substantially less than $560/ton.

Dawen Gao, Lina Du, Jiaoling Yang, Wei-Min Wu, and Hong Liang. A critical review of the application of white rot fungus to environmental pollution control. *Critical Reviews in Biotechnology*, 2010; 30(1): 70–77. This review focused on the representative strain *Phanerochaete chrysosporium* and did not include discussion of dioxins. There were no references to literature (discussed below) for strains selected for dioxin degradation.

Rodríguez-Couto S. Industrial and environmental applications of white-rot fungi. Mycosphere 8(3) 456-466 (2017) www.mycosphere.org. This paper included a very general discussion of WRF remediation as part of a larger general discussion of potential industrial applications. The paper contained no information directly relevant to WRF dioxin degradation.

2. Literature review WRF dioxins

To generate the broadest results, I conducted a review of literature from 1995 through July 2018 initially in the academic data base (Web of Science) using the key words "fungi" and "dioxin". The initial search was followed by searches using specific authors and fungal strains with a focus on studies of WRF strain selection and dioxin degradation in soils. A list of selected references is attached and copies of key papers can be provided. The search identified several review papers which included both bacterial and fungal dioxin degradation plus WRF related papers covering: enzymes and dioxin metabolic pathways, strain selection, and studies using a variety of treatment systems including defined media, solid culture media, sterile soils spiked with specific dioxin congeners and finally studies in soils from contaminated sites.

Review Papers

A review (Field and Sierra-Alvarez 2008) covered know microbial degradation pathways for PCDD and PCDF: Reductive dechlorination of rings by anaerobic bacteria, aerobic bacteria which degrade lower chlorinated compounds and white rot fungi. Aerobic bacterial pathways include both co-metabolism of dioxins with utilization of other carbon sources and some bacteria which can utilize lower chlorinated dioxins as a carbon source. The review suggests that bacterial degradation contributes to "natural attenuation of dioxin contamination". One paper cited in reviews describes dioxin biodegradation by adapting mixed cultures of bacteria (Chen et al. 2016). An earlier review (Wittich 1998) also described dioxin degradation pathways for bacteria and for different fungi. White rot fungi and some other liter and wood degrading fungi degrade dioxins via extracellular peroxidases. Some fungi, i.e. Aspergillus sp., degrade dioxins via intracellular cytochrome P-450 monooxygenase pathways. However, WRF are the subject of most literature as they degrade higher chlorinated dioxins.

A comprehensive review of WRF degradation of chloro-organic compound (Marco-Urrea and Reddy 2012) summarized enzymology of the degradation of xenobiotic contaminants in soil (and water) by extracellular peroxidases and studies on WRF degradation of chlorinated organic compounds including dioxins.

Metabolic pathways

Metabolic pathways of dioxin described by multiple authors are important, differing degradation depending on number and positions of chlorine on the ring, formation intermediates of varying toxicity and varying susceptibility to further degradation by other microbes. (Kamei and Kondo 2005, Kamei et al. 2006).

Strain selection

Multiple papers describe strain selection of WRF for dioxin degradation in the US, Japan and Europe. Most describe lab studies using liquid media or sterile soil, however several emphasize

soil conditions as a variable and screen strains grown of solid substrates such as wood chips applied to non-sterile soils. Strain selection will be a key variable in predicting fungal bioremediation of Butte Pole Yard soil. From these studies a number of species and strains stand out as having potential for dioxin bioremediation.

Early US work focused on *Phanerochaete chrysosporium* (Bumpus et al. 1985) and on strains of *Pleurotus ostreatus* Lamar 1990). Early research with these strains used liquid cultures with nitrogen limited nutrients to induce peroxidase production. Later work growing fungi on solid culture substrates and with different fungal species showed that nitrogen limitation is not a necessary condition for peroxidase production.

From multiple screening studies researchers in Japan selected strains using lab media or soil including *Bjerkandera* sp. (Sato et al. 2003), *Phlebia lindtneri* (Kamei and Kondo 2005) *Phlebia brevispora* (Kamei et al. 2006), *Coprinellus* sp. (Suhara et al. 2011) and 2 undescribed species PL 1 and 267 (Tachibana et al 2007). Tachibana studies showed up to 90% dioxin degradation as TEQ in 30 days in non-sterile soil supplemented with 0.1% surfactant. Finnish studies selected a litter degrading (but nonwhite rot) strain of *Stropharia rugosoannilata* and the WRF fungus *Phanerochaete velutina* as the most effective, reducing TEQ by 64%. Fungi were grown on pine bark then mixed in as is soil from contaminated saw mill sites (Valentin et al. 2013, Winquist et al. 2014, Anasonye et al. 2014). *Bjerkandera* was seen as advantageous as production of extracellular peroxidases occurred under nutrient sufficient conditions (Manji and Ishihara, 2004). This confirms results of Mycotech work with WRF in Butte where we saw high levels of peroxidase production and biodegradation of PCP and PAH compounds using WRF, especially *Pleurotus* sp., grown in solid substrate culture under nutrient sufficient conditions

Multiple strains show potential for dioxin degradation however many are non-indigenous to US. US strains of *Pleurotus* are promising. We did not maintain the *Pleurotus* strains used in the bioremediation tests conducted in the 1990's by Mycotech. In Earth Fax studies cited above for the New Zealand and the Darby MT sites a domestic strain of *Pleurotus* was the most effective. I don't know the status or availability of Earth Fax strains. The fungal species found effective in Japanese and European studies in the genera *Phlebia*, *Bjerkandera*, *Trametes* and *Phanerochaete* occur naturally in the US. Isolates could be obtained from culture collections or isolated from natural or contaminated habitats for screening in Butte pole yard soil.

Soil properties

Soil properties especially soil organic matter affect bioavailability and biodegradation of dioxins. Dioxins strongly sorb to soil organic matter inhibiting degradation. One study compared fungal degradation of 1,3,6,8 TCDD in extremes of three soil types, an organic rich soil, organic poor soil and soil excavated from a contaminated rice paddy. Degradation was inhibited in the organic rich soils but significantly improved with increased water content (Kamei et al. 2009). Co-cultures of different WRF (Ijoma1 and Tekere 2017) are one approach to compensating for variations in soil structure.

WRF Bioremediation

Based on WRF soil treatment methods described in literature and our own experience WRF are used in "bioaugmentation"; mixing externally grown cultures of WRF with soil in EX Situ treatment. WRF are grown on substrate materials such as wood chips or straw. When the WRF has grown through the substrate the culture is mixed with contaminated soil. The substrate provides a source of nutrition for the WRF to produce lignin degrading peroxidase enzymes and to compete with other fungi and bacteria in the soil. WRF require oxygen so depth of soil treatment is limited, generally 1-3 feet depending on soil structure. Soil is excavated, mixed with WRF culture and spread on plastic sheeting in "lifts" maybe 1 or 2 feet deep. Alternatively, soil/WRF fungal culture maybe placed in "bio piles" with active aeration, typically plastic pipe with holes in a manifold connected to fans to supply air through the soil pile. When contaminant concentration is reduced to target levels the treated soil is placed back in the excavation. In large volume sites soil may be progressively excavated and treated in units, when one unit is remediated, clean soil is returned to the excavation and a new unit excavated and treated. Depending on the quantity of fungal culture applied to soil, type and concentration of contaminates, soil conditions, temperature etc. any unit of WRF treated soil might require a few weeks to several months to reach clean up targets.

Previous WRF Treatment Test

In the early-mid 1990s the Butte based company Mycotech (I was VP for technology) had the WRF strain collection and infrastructure to conduct laboratory treatability studies and small commercial scale WRF bioremediation. We conducted multiple laboratory studies and field studies including poly aromatic hydrocarbons, PCBs, PCP and explosives. In the largest project Mycotech remediated 10,000 cubic yards of heavy hydrocarbon contaminated soil in Washington state.

Mycotech conducted a study lab and field study of WRF remediation of the Butte Pole Yard soil. At the time the focus was on the very high concentrations of pentachloro phenol, PCP, in diesel wood preservative. We adapted a strain of WRF to tolerate the high PCP concentration and used cultures of this strain grown on sugar beet pulp in on-site test plots. Test plots were monitored for PCP degradation by independent sampling, however there was no monitoring for dioxins. PCP degradation in test plots reached clean up targets in a few weeks of treatment. We proposed a WRF remediation with progressive treatment of the contaminated soil to be conducted over several summers. DEQ chose passive remediation, basically watering contaminated soil. According to the five-year review, this treatment reduced PCP to acceptable target levels. However, the PCP contained dioxins as a contaminant and treatment did not meet dioxin targets and dioxins remain the issue in about 200,000 cubic yards of contaminated soil. We did not maintain the Mycotech WRF strains used Pole yard test.

WRF Dioxin Bioremediation Butte Pole Yard

Research literature continues to show promise of WRF bioremediation and potential as an alternative treatment for the Butte Pole Yard site. However, in the literature search I did not find reports of dioxin biodegradation at the low concentrations or large soil volumes at the scale of the Butte Pole Yard. WRF bioremediation technology is site and fungal strain specific and

has not been reduced to universally applicable strains or methods for remediating dioxin contaminated soil. The best examples are studies from Finland with 64% dioxin degradation and Japan with up to 90% degradation as TEQ. These are lab studies using nonsterile contaminated soil sampled from dioxin contaminated sites.

The literature search and our experience raise a fundamental question; is treatment technology an all or nothing choice? Would 65% dioxin biodegradation reduce toxicity and risk of capping soil with dioxin left in place?

The approach to dioxin remediation at the Butte Pole Yard would treat contaminated soil with culture of a selected strain of WRF on a low cost, locally available, solid substrate. Two potential substrates are hybrid poplar from the Missoula sewage treatment plantation and sugar beet pulp from sugar mills in Billings or Sidney. In work in the 1990's Mycotech had very good results using sugar beet pulp which provided very rapid fungal growth and high levels of extracellular peroxidases (Bradley et al. 2002, 1996a 1996b). Basically, a liquid culture at about 5% v/w is used to inoculate the solid substrate which is applied to contaminated soil at a rate of about 5% to 10% v/v fungal culture to soil. We found thorough mixing of fungal colonized substrate with optimal peroxidase titer was most effective (other studies used alternating layers of culture and soil or fungi in tubes etc.). Soil would be excavated and treated in lifts of maybe 12 to 18 inches deep or in simple, aerated bio piles.

Site specific treatability studies screening strains in representative samples of Butte Pole yard soil would be the first step in assessing feasibility. This would be followed by test plots on site. More detail for design and estimated time and cost for lab treatability and field test are in a later section. Cost of laboratory treatability study and field test to evaluate feasibility of WRF dioxin remediation as a permanent solution would be small compared to overall project costs.

Full scale remediation

With successful field test results, work could proceed to full scale remediation. One issue is infrastructure for large scale fungal culture and application. At a 10% inoculation rate treatment of the pole yard soil would require about 20,000 cubic yards of fungal culture produced in batch sizes to match progressive oil treatment. For example, progressively treating contaminated soil over several summers in 50, 4,000 cubic yard increments would require fungal production of 400 cubic yards capacity. Montana BioAgriculture Inc. does not have fungal culture capacity in place at this scale; however, this is a relatively small size and this infrastructure could be readily developed in Butte (or elsewhere in Montana). Fungal production capacity might be available through Earth Fax or other companies. In addition to fungal culture, contract work would be required for soil excavation and mixing with fungal culture. Fungal strains and Montana based commercial scale fungal culture capacity developed for Butte Pole Yard could be used in remediating other sites in Montana such as the Smurfit Stone Container site near Missoula.

Based on our previous experience with large scale culture of fungi using solid substrates, a rough estimate for cost of WRF fungal culture produced locally on local substrates is in the range of

$100 per cubic yard of culture. At a 10% application rate the 20,000 yards of needed fungal culture would cost about $2,000,000. This would not include cost for soil excavation, mixing fungal culture or constructing bio piles. However, if effective WRF treatment could be cost effective compared with soil capping and would provide a permanent solution.

Neighborhood Impact

WRF bioremediation would have minimal impact on the surrounding neighborhood. Impacts of soil re excavation and handling would be short term. Excavation might generate dust but could be readily mitigated by maintaining soil moisture. Noise from excavation machinery would also be an issue but mitigated by limiting the time of work. White rot fungal cultures generate minimal odor. WRF cultures mixed in soil would be contained and after treatment is complete would simply biodegrade in place. Carbon and nutrients form the culture would improve soil fertility and aid plant growth and regeneration of the site. The site is fenced to limit access and human exposure, however any exposure to the fungal species use in remediation would not affect human health. Fungal species would be naturally occurring in wood decay in Montana and not toxic or pathogenic. In the long-term complete dioxin biodegradation would do more to ensure limited human exposure to dioxins compared to leaving dioxin in place with soil capping and institutional controls.

Site specific treatability and field study

Successful WRF dioxin bioremediation at the pole yard will depend on two factors: selection of an effective WRF strain; and site-specific soil conditions. Application rate of WRF fungal culture will determine time required for remediation and is an important component of cost. Laboratory treatability studies and field tests can determine feasibility of WRF remediation in meeting target dioxin concentrations and in predicting application rates, treatment time and cost for full scale site remediation. Tasks, timelines and costs for studies described below are estimates which can be modified based on discussions with CTEC, Butte Silver Bow government, DEQ and other interested parties. Cost for external dioxin assays would need to be determined.

Laboratory treatability study

Objectives:

1. Select effective WRF strain
2. Define WRF culture substrates and conditions
3. Estimate fungal culture application rates and incubation time
4. Determine feasibility of WRF bioremediation to meet dioxin degradation targets

All tasks would be conducted using:

nonsterile pole yard soil,
domestic, naturally occurring fungal strains,
treatments in duplicate with duplicate samples and dioxin assays.

Task 1 Baseline culture work
Evaluate five fungal strains selected from literature and obtained from culture collections.
Evaluate culture production variables, three culture substrates with variable nutrient regimes.
Establish strains and culture methods for soil treatment experiments.

Assess growth and colonization of culture substrate over time
Assay for peroxidase production

Task 2 Strain selection
Test five strains grown as per baseline culture work at high 20% dose in treatment of one composite representative pole yard soil sample. External assay for dioxin degradation at single timepoint assessed from soil colonization, peroxidase assays. Select best strain for more detailed treatment study.

Task 3
Test best strain and culture conditions, determine feasibility of WRF bioremediation to meet dioxin clean up targets.

Pole yard soil samples representing range of Pole Yard dioxin concentrations and soil conditions. Estimate five soil samples.

Test treatment using the best strain and culture conditions from tasks 1 and 2. Test at 3 different application rates, 5%, 10%, 20%. Sample at intervals, external dioxin assay to assess degradation rate and extent.

Results:
Determine feasibility of WRF bioremediation to meet clean up target,
Preliminary estimate for treatment cost
Design field test

Milestone: Recommendation to proceed

Estimated time and cost laboratory treatability study:
Time: 6 to 9 months depending on soil treatment time
Cost: MBAI labor, materials supplies: $60,000, plus cost for external dioxin assays, $5,000??

Field test
Objectives:
1. Determine feasibility of WRF dioxin bioremediation under field conditions
2. Provide data necessary to propose full scale WRF bioremediation of the Butte Pole Yard site.

Test fungal strain, culture conditions, and soil treatment conditions determined from lab treatability study under field conditions. Test plots selected to represent variations in contaminant concentrations and conditions, soil volume, sampling intervals to be determined in consultation with DEQ, CTEC. A field test might be designed with duplicate test plots of one cubic yard for each treatment at each of three to five locations on the site.

Soil excavation and handling contracted or managed by DEQ

Two treatment designs, 1) soil in lifts at variable depth 2) aerated bio piles

Results:
Demonstrate feasibility of pole yard dioxin WRF bioremediation under field conditions
Decision to proceed with full scale remediation
Detailed proposal for full scale WRF remediation of Butte Pole yard.

Estimated time and cost for field test
Time: 6 to 9 months including site soil preparation and fungal culture production. Field test timing depends on results of lab studies however fungal culture application rates and treatment conditions designed for no more than 4 to 6 months because of seasonal constraints at the Butte site.

Estimated Cost: Cost will depend on the number and size of test plots, quantity of fungal culture needed, sampling and treatment time. A reasonable estimate is:

MBAI labor, materials and supplies for fungal culture production, application and participation in site monitoring: $40,000

Soil excavation and handling: to be determined, $3,000 to $5,000?

External monitoring sampling and dioxin assays: to be determined $4,000 to $6,000?

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